

REMARKS

Claims 11-15 and 17 are canceled herein. Claim 16 is amended and new claims 19-21 are added. Support is found, for example, in the original claims.

I. Petition for Withdrawal of the Finality of the Office Action dated November 8, 2007

Applicants note that a Petition to Withdraw Finality of the Action dated November 8, 2008 was previously timely submitted on January 8, 2008, but a decision has not yet been mailed. Applicants respectfully request withdrawal of the finality of the Office Action dated November 8, 2007 for the reasons set forth in the Petition filed on January 8, 2008, which is incorporated in its entirety herein by reference.

II. Response to Claim Rejections under 35 U.S.C. § 102

A. Kammer

On page 2 of the Office Action, the Examiner has indicated that claims 11 and 13-17 remain rejected under 35 U.S.C. § 102(b) as being anticipated by Kammer (PTO-1449, 6/24/05).

Claims 11, 13-15 and 17 are canceled herein, thereby rendering the rejection as to these claims moot.

Claim 16 is amended herein by deleting the recitation of arthreostitis and rheumatoid arthritis, thereby rendering the rejection as to claim 16 moot.

Accordingly, Applicants respectfully request withdrawal of the §102 anticipation rejection.

B. Watkins

On page 4 of the Office Action, the Examiner has rejected claims 11 and 13-18 under 35 U.S.C. § 102(b) as being anticipated by Watkins (WO 02/30879 A2).

Claims 11, 13-15 and 17 are canceled herein, thereby rendering the rejection as to these claims moot.

Claim 16 is amended herein by deleting the recitation of arthreostitis and rheumatic arthritis, thereby rendering the rejection as to claim 16 moot.

Applicants provide the following with respect to claim 18 and new claims 19-21.

On pages 110-111, Watkins discloses:

The compounds of the present invention may also be used in the treatment of conditions which are known to be mediated by HDAC, or which are known to be treated by HDAC inhibitors (such as, e.g., trichostatin A). Examples of such conditions include, but are not limited to the following:
Cancer (see, e.g., Vigushin et al., 2001).
Inflammatory disease (e.g., osteoarthritis, rheumatoid arthritis) (see, e.g., Dangond et al., 1998; Takahashi et al., 1996).

Thus, while the Watkins reference may describe an activity to inhibit the HDAC activity (Primary Assay) and an activity to inhibit cell proliferation (Secondary Assay), Watkins does not have Examples or an explanation other than the disclosure above as to whether the HDAC inhibitors can be used for inflammatory disease and osteoarthritis.

In this regard, the references cited by the Watkins reference have the following disclosure (copies of the cited references are attached).

(1) Dangond et al., "Differential display cloning of HDAC3 cDNA from PHA-activated immune cells", 1998, Biochem. Biophys. Res. Commun., Vol 242, No.3, ppp. 648-652 (Attachment 1).

This reference reports the distribution of mRNA expressions of HDAC as well as influences on the various types of immune factors. At the right column on page 651, it is described, "The strong evolutionary conservation of HDACs suggests they play a fundamental

role in multiple and complex cellular pathways of immune system regulation, including cell activation, growth and differentiation, and thus represent potential molecular targets for the treatment of cancer and autoimmunity." However, this reference does not disclose whether it can be used for inflammatory disease and osteoarthritis.

(2) Takahashi et al., "Selective Inhibition of IL-2 Gene Expression by Trichostatin A, a Potent Inhibitor of Mammalian Histone Deacetylase" 1996, J. Antibiot. (Tokyo), Vol. 49, No.5, pp. 453-457) (Attachment 2).

This reference discloses that Trichostatin A, which is an HDAC inhibitor, inhibits the IL-2 gene expression and has an immunosuppressive activity and an antiproliferative activity. The *in vivo* mouse delayed type hypersensitivity (DTH) experiment confirms the immunosuppressive activity. However, this reference has no disclosure about the use of the compound for inflammatory disease and osteoarthritis.

Accordingly, Watkins and the references cited in Watkins do not concretely disclose that the HDAC inhibitors can be used for the treatment of inflammatory disease and osteoarthritis, so that there is no teaching or suggestion in these references that would lead one of ordinary skill in the art to the presently claimed invention. In addition, these references are not enabling for the treatment of inflammatory disease and osteoarthritis.

Accordingly, the rejections of the amended claim 16 and the claim 18 based on Watkins under 35 U.S.C. §102(b) should be withdrawn. Also, new claims 19-21 depend from claim 18 and are patentable for at least the same reasons as claim 18.

Accordingly, Applicants respectfully request withdrawal of the §102 anticipation rejection.

III. Conclusion

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

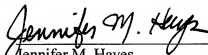
Respectfully submitted,

SUGHRUE MION, PLLC
Telephone: (202) 293-7060
Facsimile: (202) 293-7860

WASHINGTON DC SUGHRUE/265550

65565

CUSTOMER NUMBER


Jennifer M. Hayes
Registration No. 40,641

Date: January 31, 2008

Differential Display Cloning of a Novel Human Histone Deacetylase (HDAC3) cDNA from PHA-Activated Immune Cells¹

Fernando Dangond,*² David A. Hafler,* Jeffrey K. Tong,† Jeffrey Randall,‡
Ryoji Kojima,‡ Nalan Utku,‡ and Steven R. Gullans†

*Department of Neurology and †Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115; and ‡Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138

Received December 23, 1997

The nucleosomal histones can be modified through reversible acetylation by histone acetyltransferases (HATs) and deacetylases (HDACs). HATs induce nucleosomal relaxation and allow DNA-binding by transcriptional activators. HDACs form corepressor complexes which negatively regulate cell growth. However, the HDAC inhibitors butyrate, and Trichostatin A block T cell proliferation, suggesting that not all effects of HDACs lead to repression. Using mRNA differential display and 5'RACE we isolated human HDAC3, a novel gene that is upregulated in PHA-activated T cell clones. HDAC3 is homologous to other human HDACs and yeast *RPD3*. In peripheral blood mononuclear cells (PBMCs), activation by PHA, PMA and α -CD3 increased HDAC mRNA but no effect was seen with IFN- γ , LPS, or IL-4. In contrast, GM-CSF downregulated PBMC levels of HDAC3 mRNA. All HDACs were found to be ubiquitously expressed in immune and non-immune tissues. In human myeloid leukemia THP-1 cells, HDAC3 transfection resulted in increased size, aberrant nuclear morphology and cell cycle G2/M cell accumulation. Functional activity of the expressed HDAC3 protein was confirmed in α -HDAC3 antibody immunoprecipitates by a histone deacetylase assay. Our study suggests the participation of HDACs in cell cycle progression and activation. © 1998 Academic Press

The core histones are structural components of nucleosomes that play key regulatory functions, as their N-terminal domains are post-translationally

modifiable through reversible acetylation of their lysine residues [1, 2]. The dynamic equilibrium of lysine acetylation depends on two groups of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). Hyperacetylated histones decondense chromatin and make the DNA accessible to transcriptional activation complexes [3, 4]. Deacetylation of histones counteracts this effect by limiting accessibility of activation factors [5, 6] and allowing the binding of known transcriptional repressor complexes to DNA, of which HDACs themselves are part [7, 8].

Recent work identified two human HDACs [9, 10] that form molecular repressor complexes by associating with molecules such as Mad [11], mSin3 [12, 13], YY1 [10] and the nuclear receptor corepressor N-CoR [8]. In addition, studies with HDAC inhibitors such as butyrate and trichostatin A (TSA) substantiated the central role of histone acetylation in gene transcription and differentiation [14-17]. Furthermore, these inhibitors induce specific cell cycle arrest at G1 and G2 phases [17], highlighting the complex regulatory role played by HDACs.

In the present study we used mRNA differential display of PHA-stimulated T cell clones and 5'RACE to isolate HDAC3, a novel HDAC gene whose expression in immune cells appears to be regulated during activation. Moreover, HDAC3 overexpression disturbs cell cycle progression in human cells.

MATERIALS AND METHODS

Isolation of T cell clones. Myelin basic protein (MBP) reactive T cell clones isolated by limiting dilution have been extensively characterized [18-20]. CD4⁺ T cell clones Ob1A12.8, Ob3D1.7, HyG11.8 and A182 are DR2 restricted and recognize epitope MBP₁₉₋₃₃; the HTLV-I reactive CD8⁺ T cell clone JS-2E11.7 is A2 restricted and directed against epitope Tax₁₁₋₂₁ [21].

Studies with activation and growth factors. PBMCs were isolated from healthy donors using a Ficoll-paque (Pharmacia) gradient cen-

¹ The sequences in this paper have been deposited in GenBank (Accession No. U66914).

² Corresponding author. Department of Neurology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115. Fax: (617) 525-5711. E-mail: dangond@wch.harvard.edu.

trifugation. Cells were cultured in RPMI supplemented with 10% human AB serum, 10% IL-2-containing T-Stim (Collaborative Biomedical Products), 2 mM L-glutamine, 10 mM Hepes, and 100 U/100 µg per ml penicillin/streptomycin in Petri dishes at 1×10^6 cells/ml. To assess the response of HDACs to growth and differentiation inducers, PBMCs were stimulated for 48 hours with rIL-4 (Boehringer Mannheim) at 10 ng/ml, rIFN- γ (Gibco BRL) at 10 ng/ml, or GM-CSF (Pernogen) at 5 ng/ml. To assess the response to polyclonal activation, we used PHA (Sigma) at 10 ng/ml, PHA (Murex Diagnostics, Dartford, UK) (1 µg/ml), or α -CD3 (OKT3) Ab (Ortho Biotech, Inc., Raritan, NJ) at 1 ng/ml. Petri dishes had been coated with α -CD3 (1 ng/ml) the night prior and washed twice with PBS, before adding the cells. LPS (Sigma) was used at 10 µg/ml as a control for rIFN- γ -induced activation. Cells were recovered after 48 hours by mechanical dislodging and viability (>95%) was assessed by trypan blue exclusion. Human myeloid leukemia THP-1 cells were grown in RPMI media supplemented with 10% FCS, 10 mM Hepes, 2 mM L-glutamine, and 100 U/100 µg per ml penicillin/streptomycin.

RNA isolation and Northern analysis. Total RNA was isolated from T cell clones and PBMCs using the RNeasy B method (Tel-Test, Inc.). Northern blots with 20 µg of total RNA per lane were prepared and probed as described [22]. The blots were washed twice at room temperature (2x SSC, 0.1% SDS, 20 min), once at 60°C (0.2x SSC, 0.1% SDS, 20 min), and autoradiographed. An L3 ribosomal cDNA probe was used to verify equivalent loading. Blots containing poly(A)⁺ RNA (2 µg) were purchased (Clontech) and probed for tissue distribution studies. Hybridizations with either the 343 bp cDNA fragment or the R98879 EST insert yielded identical results. The HDAC1 cDNA probe was kindly provided to us by Stuart Schreiber and Christian Hassig (Harvard University). From Genome Systems (St. Louis, MO) we obtained the cDNA for HDAC2 (EST R06693) to use as a probe. We used a human p53 cDNA probe obtained by PCR as a positive control for rIFN- γ -induced activation [23].

Isolation of HDAC3. mRNA differential display was performed as described [22] using the primers 5'-GATGCCAGCATGG-3' and 5'-AATAACGCCATT-3'. The differentially expressed cDNA fragment was excised and eluted from the gel, reamplified, cloned into a TA vector (Invitrogen) and sequenced. An identical cDNA (EST R98879) was identified by a dbEST database search and obtained from Genome Systems. The missing 5' end of the cDNA was obtained by performing 5'RACE (Gibco, BRL) and the full-length cDNA was constructed using an overlapping PstI restriction site and ligation into the pCDNA3.1.2eo vector (Invitrogen). Sequencing was performed at the Howard Hughes Biopolymers Research Facility at Harvard Medical School.

HDAC3 transfection. The pCDNA3.1.2eo and the pCDNA3.1.2eo-HDAC3 constructs were transfected into THP-1 cells by electroporation with a Biorad electroporator (300 V and 1,000 microFarads). Stable transfectants were selected with Zeocin-containing media. Changes in morphology were observed with a Nikon phase contrast microscope.

CC analysis and flow cytometry. Cells were fixed in 70% ethanol and Propidium iodide (PI) was added following the protocol described by Nicoletti [24], with minor modifications. Analysis of cellular DNA content was performed in a Beckton Dickinson FACS sorter.

Histone deacetylase activity assay. For the HDAC activity assay, THP-1 cells were grown and collected 2 days after PHA stimulation. Cells were spun down, washed with ice cold PBS, spun down again at 1000 x g for 5 minutes. The cells were then lysed in 400 µl lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 0.2 mM PMSF) for 30 min at 4 °C with gentle inversion. Lysates were centrifuged at 14,000 x g for 2 min to pellet insolubles. The supernatants were collected and aliquoted in 2 tubes each. Rabbit α -HDAC3 antisera (30 µl, kindly provided by Christian Hassig and Stuart Schreiber,

Harvard University) was added to each tube followed by 1 hr incubation on ice. Protein A agarose (40 µl) (Gibco, BRL) was added to each tube and tubes were rotated at 4 °C for 3 hr. Beads were then washed 3x with 500 µl lysis buffer. Beads were then resuspended in 200 µl histone deacetylase buffer (50 mM Tris pH 7, 150 mM NaCl, 10% glycerol, 1 mM EDTA). This suspension was then split to 2 tubes of 100 µl each. Trichostatin A (Wako Pure Chemicals) was added to 200 nM final concentration, followed by incubation on ice for 15 minutes. ³H acetylated histone substrate was then added to all tubes and reactions proceeded at 37 °C with vigorous shaking for 3 hr. Reactions were terminated by the addition of 50 µl 0.1 M HCl, 0.16 M AcOH followed by 800 µl ethyl acetate. Tubes were vortexed and spun in a microfuge to separate aqueous layers. The organic phase (400 µl) was added to 3 ml scintillation fluid (DuPont EcoScint) and counted in a scintillation counter (Beckman).

RESULTS AND DISCUSSION

Using mRNA differential display we identified a cDNA present in four chronically PHA-activated T cell clones (Ob1A12.8, Hy3D1.7, HyG11, and KS.2E11.7) but not present in a freshly isolated T cell clone (A192) that had undergone fewer rounds of PHA stimulation. This 343 bp cDNA fragment was cloned, sequenced, and compared to the NCBI-GenBank database using BLASTX. The deduced amino acid sequence was found to have significant homology to the amino terminus of the two known human HDAC cDNAs and the mouse HDAC1 and yeast RPD3 cDNAs suggesting it encoded a new family member. Comparison to the Expressed Sequence Tag (EST) database revealed several identical overlapping human cDNAs. From Genome Systems Inc. (St. Louis, MO) we obtained a 1.8 kb human EST cDNA clone (#R98879) whose 5' end overlapped with our clone. We performed 5'RACE on mRNA from PHA-stimulated PBMCs and obtained the remaining sequence and an additional 75 nt of the 5' UTR such that the full-length HDAC3 cDNA is 1984 bp long with an ORF of 1284 nt.

The deduced 428 amino acid sequence of HDAC3 (Fig. 1A) predicts a molecular mass of 48,750 Da. It is 50-53% identical to the human HDAC1 [9], HDAC2 [10], and the RPD3 gene from yeast [25]. The homology tree analysis of the protein sequences (Fig. 1B) revealed that human HDAC3 is more similar to yeast RPD3 than other human HDACs or the other murine, frog, and *Drosophila* homologs. The sequence homology among HDACs spans most of the molecule but the carboxyl-terminus is non-conserved, highly charged and enriched in glutamate and aspartate residues. Hydrophobicity analysis using the Kyte-Doolittle algorithm confirmed the structural similarity among the human and yeast HDACs (Fig. 1C).

To identify tissues that express HDACs, we probed northern blots of immune and non-immune tissues. All HDACs were ubiquitously expressed (Fig. 2) but the levels in non-immune tissues was less uniform, with the highest relative levels of HDAC3 in heart. Thus, the HDACs have tissue-specific expression profiles, but

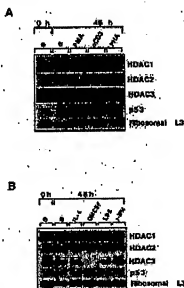


FIG. 3. Northern analysis of peripheral blood mononuclear cells (PBMCs) shows that HDAC mRNA is modified by polyclonal activators and GM-CSF. A. PBMCs were cultured with or without PMA, PHA, or α -CD3. B. HDACs expression is downregulated by GM-CSF at 48 hours but not significantly altered by γ -IPN, IL-4 or LPS. Not shown, the downregulation by GM-CSF was seen at earlier time points (8 and 24 hours) for HDAC1 and 2 but not for HDAC3. A p33 cDNA probe was used as a control in (A) and (B). Regulation of HDACs by polyclonal activators and GM-CSF was confirmed by analyzing PBMCs mRNA from three healthy donors in three separate northern blot experiments.

increase in cell size and aberrant nuclear morphology (not shown) suggesting an inability of these cells to progress coordinately and undisturbed through the cell cycle and a poor integration of size control mechanisms. Our results are consistent with the G2/M cell cycle abnormalities observed when transfecting mouse HDAC1 into Swiss 3T3 cells [29]. Of note, both in plant [31] and yeast [32], modification of histones to prevent ac-

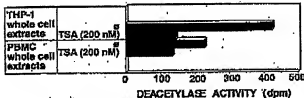


FIG. 4. Histone deacetylase activity assay. Human THP-1 myeloid leukemia cells and PHA-stimulated PBMCs were collected after 48 hrs in culture media. Whole cell extracts were analyzed in duplicate for ability to deacetylate [3 H]acetylated histone substrates, and [3 H]acetic acid release was expressed as the average in dpm.



FIG. 5. FACS analysis of DNA content reveals cell cycle abnormalities induced by HDAC3 overexpression. Stably-transfected THP-1 cell lines exhibit decreased accumulation in the G0/G1 phase, no increase in the relative percentage of cells in the early S phase, and a distinct accumulation of cells in the G2/M phase (three independent experiments yielded consistent findings). Relative percentages of cells in G2/M are shown. Necrotic cells were excluded from the analysis. Ten thousand cells were counted for each sample.

tylation results in G2/M abnormalities, suggesting a role for HDACs in modulation of the mitotic stage.

Identifying the transcriptional "switches" that define the phenotype of activated immune cells will likely provide a better understanding of the molecular mechanisms underlying immunity. Histone acetylation and deacetylation are important determinants of gene transcription and cell differentiation. The strong evolutionary conservation of HDACs suggests they play a fundamental role in multiple and complex cellular pathways of immune system regulation, including cell activation, growth and differentiation, and thus represent potential molecular targets for the treatment of cancer and autoimmunity. Detailed studies to define the specific immune cell types involved in HDAC upregulation upon activation and the mechanisms responsible for HDAC-induced cell cycle abnormalities are now under way.

ACKNOWLEDGMENTS

The authors thank Drs. Bento Santos and Alejandro Chavakis for helpful suggestions and technical help, and Dr. Luca Ammend for providing an antigen-reactive T cell clone derived from a normal individual. This work was supported by a Howard Hughes Physician Scientist Post-doctoral Fellowship (F.D.), a NSF Pre-doctoral Fellowship and the Harvard-Markey Biomedical Scientist Program (J.K.T.), and National Institutes of Health Grants DK6031 (S.R.G.) and NS24247 (D.A.H.).

REFERENCES

1. Johnson, L. M., Kayne, P. S., Kahn, E. S., and Grunstein, M. (1989) *Proc Natl Acad Sci USA* 87, 6286-6290.
2. Mege, P. C., Morgan, B. A., Mittman, B. A., and M. M. S. (1990) *Science* 247, 841-845.
3. Hobbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1990) *EMBO J.* 7, 1395-1402.
4. Lee, D. Y., J. J., H., Fries, D., and Wolf, A. F. (1993) *Cell* 72, 73-84.

5. Roth, S. Y., and Allis, C. D. (1996) *Cell* 84, 843-851.
6. Wolffe, A. P., and Pruss, D. (1996) *Cell* 84, 817-819.
7. Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D., and Bröschi, J. R. (1993) *Genes Dev.* 7, 592-604.
8. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Söderström, M., Laherty, C. D., Torchia, J., Yang, W.-M., Brard, G., Ngo, S. D., Davis, J. R., Seto, E., Eisenman, R. N., Rhee, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* 387, 43-48.
9. Tamton, J., Hassig, C. A., and Schreiber, S. L. (1990) *Science* 272, 408-411.
10. Yang, W.-M., Inouye, C., Zeng, Y. Y., Beasry, D., and Seto, E. (1995) *Proc. Natl. Acad. Sci. USA* 92, 12845-12850.
11. Laherty, C. D., Yang, W.-M., Sun, J.-M., Davis, J. R., Seto, E., and Eisenman, R. N. (1997) *Cell* 89, 349-358.
12. Hassig, C. A., Fleischer, T. C., Eddin, A. N., Schreiber, S. L., and Ayer, D. E. (1997) *Cell* 89, 341-347.
13. Zhang, Y., Irami, R., Erdjument-Bromage, H., Tempst, P., and Reuberg, D. (1997) *Cell* 89, 357-364.
14. Van Lint, C., Emiliani, S., Ott, M., and Verdine, E. (1996) *EMBO J.* 15, 1112-1120.
15. Hoshikawa, Y., Kwon, H. J., Yoshida, M., Horinouchi, S., and Beppu, T. (1994) *Exp. Cell Res.* 216, 189-197.
16. Candito, E. P., Reeves, R., and Davis, J. R. (1978) *Cell* 14, 105-113.
17. Yoshida, M., Horinouchi, S., and Beppu, T. (1995) *Bioessays* 17, 423-430.
18. Ota, K., Matsui, M., Milford, E. L., Mackin, G. A., Weiner, H. L., and Hadler, D. A. (1990) *Nature* 346, 183-187.
19. Wucherpfennig, K. W., Sette, A., Southwood, S., Omsuff, C., Matsui, M., Strominger, J. L., and Hadler, D. A. (1994) *J. Exp. Med.* 179, 279-290.
20. Wucherpfennig, K. W., and Strominger, J. L. (1995) *Cell* 80, 695-705.
21. Hållsberg, P., Weber, W. E. J., Dargatzis, F., Butra, V., Sette, A., and Hadler, D. A. (1995) *Proc. Natl. Acad. Sci. USA* 92, 4086-4090.
22. Kojima, R., Randall, J., Brenner, B. M., and Cullis, S. R. (1996) *J. Biol. Chem.* 271, 12327-12332.
23. Kato, A., Watanabe, Y., Takada, N., Aizawa, S., and Aikawa, T. (1997) *J. Biochem. (Tokyo)* 121, 677-683.
24. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991) *J. Immunol. Methods* 139, 271-276.
25. Vidal, M. S., and Gaber, R. F. (1991) *Mol. Cell Biol.* 11, 6317-6327.
26. Mateo, M. S., Sanchez-Busto, M., Martínez, J. C., Orfao, A., Oradire, J. L., and Piris, M. A. (1995) *J. Clin. Pathol.* 48, 151-159.
27. Reed, J. C., Alpers, J. D., Nowell, P. C., Hoover, R. G. (1986) *Proc. Natl. Acad. Sci. USA* 83, 3982-3986.
28. McCubrey, J. A., Steelman, L. S., and McKernan, J. P. (1991) *Oncogene Res.* 6, 1-12.
29. Bard, S., Topick, J., Laggar, C., Khien, H., Kuchler, K., and Soler, C. (1997) *Mol. Cell Biol.* 17, 5033-5043.
30. Soehnle, M. A., Connors, S. A., Sullivan, R., Elias, A., Antman, K., Schnipper, L., and Griffin, J. D. (1988) *Blood* 72, 691-697.
31. Belyaev, N. D., Houben, A., Baranczewski, P., and Schubert, I. (1997) *Chromosoma* 106, 193-197.
32. Kaye, P. S., Kim, U.-J., Han, M., Mullen, J. R., Yoshizaki, F., and Grunstein, M. (1995) *Cell* 85, 27-39.

Selective Inhibition of IL-2 Gene Expression by Trichostatin A, a Potent Inhibitor of Mammalian Histone Deacetylase

ISAMI TAKAHASHI*, HIROMASA MIYAJI, TETSUO YOSHIDA,
SOICHIRO SATO[†] and TAMMO MIZUKAMI

Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
3-6-6 Arahimachi, Machida-shi, Tokyo 194, Japan.

[†]Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd.,
1188 Shimotogari, Nagatsumi-cho, Shimizu 411, Japan.

(Received for publication October 6, 1995)

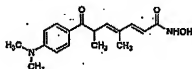
During screening for inhibitors of T cell activation, we have found that trichostatin A (TSA), known as a potent inhibitor of histone deacetylase, showed selective inhibitory activity against IL-2 gene expression. From luciferase reporter experiments on human leukemic Jurkat T cells, TSA was found to inhibit the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter with a 50% inhibitory concentration value of 0.073 μ M. On the other hand, TSA, at the same concentration, enhanced the expression of the luciferase reporter gene directed by the *c-fos* enhancer and promoter. The result of RT-PCR experiments also indicates that TSA has selective inhibitory activity against IL-2 gene expression in Jurkat cells. These results suggest that the change in chromatin structure caused by the hyperacetylation of histone might affect the regulation of IL-2 and *c-fos* gene expression.

Activation of T lymphocytes is a complex process requiring recognition of the antigen by the T cell antigen receptor as well as concomitant costimulatory signals from antigen presenting cells. These events induce the activation of second messenger pathways such as tyrosine kinases and protein kinase C and lead to an increase in intracellular Ca^{2+} . These signals finally give rise to a sequential activation of sets of genes that in turn initiate proliferation, differentiation and immunological functions¹⁾. Although detailed mechanisms for these events are still unclear, recent studies concerning the molecular actions of cyclosporin A (CsA)²⁾ and FK506 have provided important new information on the biochemical process which regulates these events. Of special significance has been the realization that these molecules, act as "molecular adaptors" which serve to mediate the interaction between their respective intracellular drug-binding proteins (or "immunophilins") and their individual target molecules. Vigorous research on the mode of action of CsA and FK506 has shown that the heterodimeric, Ca^{2+} /calmodulin-regulated phosphatase calcineurin is a major common target of the CsA-cyclophilin A and FK506-FK506 binding protein 12 (FKBP12) drug-immunophilin complex *in vitro* and that drug-immunophilin complex blocks the dephosphorylation by calcineurin of the nuclear factor of activated T cells (NFATc) in cytoplasm, a step that is required for its translocation to the nucleus. The drug-immunophilin

complexes with calcineurin and inhibition of its phosphatase activity provide a molecular basis for the inhibitory effect of CsA or FK506 on expression of gene encoding IL-2 and other cytokines³⁾. Although several signaling inhibitors have been reported for the effect of the gene expression, as of now no selective inhibitor of IL-2 gene expression is known other than CsA and FK506 (manuscript in preparation).

Trichostatin A (TSA), which had been originally found from its antifungal activity⁴⁾, was shown by YOSHIDA *et al.* to induce differentiation of Friend leukemia cell and to inhibit the cell cycle of normal rat fibroblasts in the G1 and G2 phases at low concentration⁴⁻⁶⁾, and shown by SUGITA *et al.* to induce morphological reversion of *sis*-transformed NIH3T3 cells⁶⁾. YOSHIDA *et al.* also found that the target molecule of TSA was histone deacetylase that is potently and specifically inhibited by

Fig. 1. Structure of trichostatin A.



Trichostatin A

TSA⁷. Since histone proteins have an essential supporting role in the transcriptional machinery for regulating gene expression, the relationship between core histone hyperacetylation caused by the inhibition of the histone deacetylase and several gene expressions is receiving increasing attention⁸. It has been reported that TSA which induces histone hyperacetylation affects the gene expression of gelolin⁹, histone H1^{10,10}, cytokeratin A (endo A)¹¹ and early gene products (*c-fos*, *c-fos* and *c-myc*)¹².

We report here that TSA shows inhibitory activity against the IL-2 gene expression and enhancing activity against the *c-fos* gene expression, and that TSA has immunosuppressive activity in a mouse experimental model.

Materials and Methods

Isolation of Trichostatin A (TSA)

A reporter gene assay, which is luciferase assay described below, has been used to screen for isolation of TSA. The TSA producing organism was isolated from soil collected in Japan and was taxonomically classified as *Streptomyces* sp. GT15. TSA was accumulated in both the mycelium and culture filtrate. After adjustment to pH 4.0 with 6N HCl, the culture filtrate (30 liter) was applied to a column of Diaion HP-20 (2 liter) (Mitsubishi Chemical Industries Limited). The column was washed with deionized water and 30% methanol (MeOH) and then eluted with 100% MeOH. After concentration, the eluate was extracted with normal-butyl alcohol (*n*-BuOH). The extract was concentrated and the residue was subjected to silica gel (Merck Art. No. 7734) column chromatography using the stepwise method of chloroform (CHCl₃)-MeOH as eluting solvents. The active fractions were combined and evaporated to dryness. The residue was rechromatographed on silica gel (Merck Lichroprep Si 60) with CHCl₃-MeOH, and the active fractions were further purified with HPLC using a packed column (YMC-ODS SH-365-S, 65% MeOH) to yield 63 mg of TSA.

Cell Lines and Culture

Jurkat cells were maintained in complete RPMI1640 (Gibco) supplemented with 10% (v/v) fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml) in a 5% CO₂ air atmosphere. Jurkat/pIL2uc2 #15 and Jurkat/pfosuc22 #39 were periodically cycled in the above media with 0.3 mg/ml hygromycin B.

Plasmid Construction and Luciferase Assays

Plasmid construction, transfection and selection of hygromycin-resistant Jurkat clones were described by Miyai *et al.* (manuscript in preparation). Jurkat/pIL2uc2 #15 cells were stimulated in 200 μ l fresh culture media with 12-*O*-tetradecanoylphorbol-13-acetate

(TPA) (5 ng/ml) and phytohemagglutinin (PHA) (1 μ g/ml) in a tube and incubated for 6 hours at 37°C. Jurkat/pfosuc22 #39 cells were stimulated in 200 μ l fresh culture media with TPA (5 ng/ml) and PHA (1 μ g/ml) in a tube and incubated for 3 hours at 37°C. TSA was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM and used at the indicated concentrations. After incubation, luciferase activities were measured using a luminometer (EG & G Berthold Autolumat LB553).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

RNA samples were reverse transcribed with Superscript RT (Bethesda Research Laboratories, Rockville, MD) and IL-2 or *c-fos* specific primers (STRATAGENE) were used to amplify selected IL-2 or *c-fos* with AmpliTaq DNA polymerase (Cetus corporation).

Mixed Lymphocyte Reaction (MLR)

Mice were obtained from Nihon SLC. MLR were set up as previously described by Webb *et al.*¹³. 1.5 $\times 10^5$ Lymph node responder cells from B10.BR mice were cultured with 5 $\times 10^5$ mitomycin C-treated spleen stimulator cells from AKR mice. The culture was pulsed with 1 μ Ci per well of [³H]thymidine approximately 18 hours before harvesting onto fiber filter-mats, and then counted using a scintillation counter.

Antiproliferative Activity

For determination of the antiproliferative activity of TSA, Jurkat/pIL2uc2 #15 was cultured in RPMI1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified 5% CO₂ atmosphere. Cells were plated into 96-well microtiter plate (10⁴ cells/well) in the presence of various concentrations of TSA and incubated for 72 hours. Culture was pulsed with 1 μ Ci per well of [³H]thymidine 18 hours before harvesting onto filter-mats, and counted in a scintillation counter. The results were expressed as an IC₅₀, the drug concentration required for 50% inhibition of cell growth. HeLa S3 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml) and streptomycin (0.1 mg/ml). HeLa S3 cells were preincubated for 24 hours at 37°C in 96-well dishes and then treated with different dilutions of TSA for 3 days. Thereafter, the concentration of TSA required for 50% inhibition of cell growth was determined by Gimsa staining¹⁴.

Delayed-type Hypersensitivity (DTH)¹⁵

To induce DTH to trinitrophenyl (TNP), 0.1 ml of 10 mM trinitrobenzene sulfonic acid (TNBS) solution (pH 7.4) was subcutaneously injected into two separate sites on the dorsal flanks of the Balb/c mice. The compound dissolved in 10% DMSO was injected intraperitoneal (i.p.) once a day through day 0 to day 4. Ten percent DMSO was used as the control. Challenge was performed

5 days later by injecting 0.05 ml of 10 mM TNBS solution (pH 7.4) into the right footpad. Twenty-four hours after the footpad challenge, DTH reactivity was assessed by measuring the swelling of the footpad. The magnitude of the DTH was expressed as the increment of the thickness of the challenged right footpad as compared with the untreated left footpad. Each group consisted of five mice. TSA was dissolved in DMSO and administered by i.p. injection for 5 consecutive days, beginning on the day of sensitization.

Results and Discussion

In the course of screening for inhibitors of the T cell signal transduction pathway leading to IL-2 gene expression, we have found that a strain of *Streptomyces* sp. GT15 produced a compound which showed a potent inhibitory activity against the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter. The compound was isolated from the culture broth by the combination of column chromatographies, and identified, based on physico-chemical properties and NMR analysis, as trichostatin A (TSA) previously known as a potent inhibitor of histone deacetylase (Fig. 1).

From the luciferase reporter experiments on Jurkat cells, TSA inhibited the expression of the luciferase reporter gene directed by the IL-2 enhancer and promoter with the 50% inhibitory concentration (IC_{50}) value of

0.073 μ M. On the other hand, interestingly, TSA enhanced the reporter gene expression directed by the *c-fos* enhancer and promoter at the same concentration as shown in Fig. 2. These potent contrasting activities of TSA were reduced by glycosylation at the position of hydroxamate, trichostatin C⁽⁶⁾ (data not shown), indicating that the hydroxamate moiety of TSA is essential for the effects on the reporter gene expression. These results are showing that contrasting activities of TSA on gene expression of IL-2 and *c-fos* were ascribed to the inhibition of histone deacetylase. Because the inhibitory activity of TSA on present gene expression was dependent on the hydroxamate moiety which is parallel with the effect on histone deacetylase⁷ and also there was no common characteristics in the promoter-enhancer regions of the genes irrespective of different sensitivities to TSA.

It has been reported that TSA affects the gene expression of gelsolin⁹, histone H1¹⁰, cytochrome A (endo A)¹¹ and early gene products (*c-jun*, *c-fos* and *c-myc*)¹², all of these reported gene expressions, except of *c-jun*, have been significantly enhanced by TSA. Therefore, it is noteworthy that TSA selectively inhibits the IL-2 gene expression.

Before the discovery that TSA is an inhibitor of histone deacetylase, sodium *n*-butyrate was used to inhibit histone deacetylase, although its inhibitory activity is weak.¹⁷⁻²⁰ We have tried to determine whether sodium *n*-butyrate also shows selective inhibitory activity against the IL-2 gene expression and enhancement activity against the *c-fos* gene expression. Although the concentration of the drug is very high (IC_{50} = 1.0 mM), sodium *n*-butyrate inhibited the IL-2 reporter gene expression and enhanced the *c-fos* reporter gene expression like TSA (Fig. 3). While the observations shown here for sodium *n*-butyrate have not been reported, a rapid alteration of the *c-myc* and *c-jun* gene expression caused by sodium *n*-butyrate has been reported²¹.

These results indicate that core histone hyperacetylation caused by the inhibition of histone deacetylase may be involved in the alteration of IL-2 and *c-fos* gene expression.

To confirm the selective inhibitory activity against the IL-2 gene expression, the effect of TSA on the endogenous IL-2 mRNA expression on Jurkat cells was investigated using a RT-PCR experiment. As shown in Fig. 4, TSA as well as cyclosporin A (CsA) inhibited induction of endogenous IL-2 mRNA at the concentration of 1 μ M, not that of β -actin and GAPDH mRNA as controls. Similarly, induction of endogenous *c-fos* mRNA was

Fig. 2. Effect of trichostatin A on IL-2 and *c-fos* reporter expression.

IL-2 (●) and *c-fos* (■) reporter activities in the presence of various concentrations of TSA.

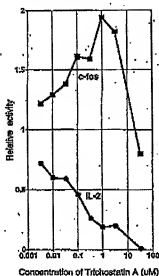


Fig. 3. Effect of sodium *n*-butyrate on IL-2 and *c-fos* reporter expression.

IL-2 (●) and *c-fos* (■) reporter activities in the presence of various concentrations of sodium *n*-butyrate.

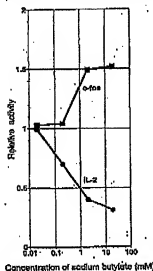
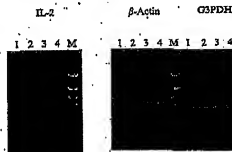


Fig. 4. Effect of trichostatin A on internal IL-2 mRNA expression by RT-PCR.

lane 1: no addition, lane 2: +TPA (5 ng/ml)+PHA (1 µg/ml), lane 3: +TPA (5 ng/ml)+PHA (1 µg/ml)+TSA (1 µM), lane 4: +TPA (5 ng/ml)+PHA (1 µg/ml)+cyclosporin A (1 µM), M: marker.



enhanced by TSA but not by CsA (data not shown). These results indicate the following. First, since the endogenous IL-2 mRNA expression as well as the IL-2 reporter expression were affected by TSA, the activities of TSA against the reporter expression do not appear to be due to the positional effect of a stable transformant of Jurkat cells. Second, the mode of action of TSA is quite different from that of CsA because of its different effect on the *c-fos* gene expression.

IL-2 production on Jurkat cells stimulated by PHA and TPA was also inhibited by TSA using enzyme linked immunosorbent assay (ELISA) (data not shown).

Table 1. Effect of trichostatin A on delayed type hypersensitivity (DTH).

Compound	Dose (mg/kg)	Inhibition (%)
Trichostatin A	100 x 5 tp	64.4 (4/Toxic)
	50 x 5 tp	47.1
	25 x 5 tp	17.2
	12.5 x 5 tp	8.7
Cyclosporin A	30 x 5 po	94.5

In order to investigate immunosuppressive activity, TSA was first assessed *in vitro* using the mixed lymphocyte reaction (MLR). As results, TSA showed inhibitory activity against the MLR with an IC_{50} value of 0.032 µM and showed antiproliferative activity against the Jurkat cells with an IC_{50} value of 0.032 µM, while the IC_{50} value of antiproliferative activity against HeLa S3, a nonlymphoid cell line, was 1.1 µM. Secondly, TSA was assessed *in vivo* using the mouse delayed type hypersensitivity (DTH) experiment. TSA showed 47.1% inhibition by i.p. injection of 50 mg/kg x 5 as shown in Table 1, although the inhibition of TSA against DTH is less effective than that of CsA (30 mg/kg x 5 p.o.; 94.5%).

TSA has a variety of biological activities such as antifungal, induction of cell differentiation, cell cycle arrest, morphological change and effect on gene expression^{2-6,9-12}. These biological activities of TSA are considered to be a result of the inhibition of histone deacetylase⁷. However, there is little information on the relation between these biological activities and inhibition of histone deacetylase on gene expression.

The level of histone acetylation is mainly controlled by the acetyltransferase-deacetylase equilibrium²³. Reversible histone acetylation, which occurs at the ϵ -amino group of specific internal lysine residues located at the concerned domains of the core histones, is supposed to play an important role in the regulation of the chromatin structure and function specifically in DNA replication and transcription²³. TSA inhibits histone deacetylase resulting in the hyperacetylation of histone⁹. The hyperacetylation of histone is generally considered to provoke relaxation of the chromatin structure to make various transcriptional factors accessible to DNA. It seems that the transient increase in the site specific or phase dependent histone acetylation may be essential for the early stage of gene expression.

Although it is not clear why the inhibition of histone deacetylase causes the inhibition of IL-2 gene expression and activation of the *c-fos* gene expression, it is of great

interest to know the mechanisms that TSA possesses for contrasting activities against immediate-early (*c-fos*) and early gene (*IL-2*) expression caused by external stimuli on Jurkat cells. The dramatic change in chromatin structure by the hyperacetylation of histones might affect the DNA binding capability of various transcriptional factors or their regulatory molecules. This might result in the difference between the *IL-2* gene expression induced only by specific stimuli from the T cell receptor and the *c-fos* gene expression induced by non-specific stimuli. Future experiments must address how a set of transcriptional factors and/or their regulatory molecules recognize the acetylated form of the chromatin structure and control the *IL-2* and/or *c-fos* gene expression to coordinate the complex organization of T cell signal transductional events.

Acknowledgments

The authors deeply thank Ms. Y. NAITOH, Ms. M. KAMIYA, Ms. K. HASEGAWA and Mr. Y. NISHIE for their technical assistance.

References

- 1) WEISS, A. & D. R. LITMAN: Signal transduction by lymphocyte antigen receptors. *Cell* 76(2): 263-274, 1994
- 2) SCHREIBER, S. L. & G. R. CRAVITZ: The mechanism of action of cyclosporin A and FK506. *Immunology Today* 13(4): 136-142, 1992
- 3) TSUJI, N., M. KOBAYASHI, K. NAGASHIMA, Y. WAKISAKA & K. KOZUMI: A new antifungal antibiotic, trichostatin. *J. Antibiotics* 29: 1-6, 1976
- 4) YOSHIDA, M., S. NIMURA & T. BEPPU: Effects of trichostatin on differentiation of murine erythroleukemia cells. *Cancer Res.* 47(14): 3688-3691, 1987
- 5) YOSHIDA, M. & T. BEPPU: Reversible arrest of proliferation of rat 3Y1 fibroblasts in both the G1 and G2 phases by trichostatin A. *Exp. Cell Res.* 177: 122-131, 1988
- 6) SUGITA, K., K. KOZUMI & H. YOSHIDA: Morphological reversion of six-transformed NIH3T3 cells by trichostatin A. *Cancer Res.* 52: 168-172, 1992
- 7) YOSHIDA, M., M. KIDMA, M. AKITA & T. BEPPU: Potent and specific inhibition of mammalian histone deacetylase both *in vivo* and *in vitro* by trichostatin A. *J. Biol. Chem.* 265: 17174-17179, 1990
- 8) WOLFE, A. P.: Transcription: in tune with the histones. *Cell* 77: 13-16, 1994
- 9) HOSHIKAWA, Y., H. J. KWON, M. YOSHIDA, S. HORNOUGH & T. BEPPU: Trichostatin A induces morphological changes and gelsolin expression by inhibiting histone deacetylase in human carcinoma cell lines. *Exp. Cell Res.* 214: 189-197, 1994
- 10) GHARADOT, V., T. RABLOU, M. YOSHIDA, T. BEPPU, J.-J. LAWRENCE & S. KHACHAT: Relationship between core histone acetylation and histone H10 gene activity. *Eur. J. Biochem.* 224: 885-892, 1994
- 11) MIYASHITA, T., H. YAMAMOTO, Y. NISHIMURA, T. MORITA & A. MATSUHARA: Activation of the mouse cytokinin A (*endo A*) gene in teratocarcinoma F9 cells by the histone deacetylase inhibitor Trichostatin A. *FEBS Lett.* 353: 225-229, 1994
- 12) PUTAMURA, M., Y. MONDEN, T. OKABE, J. FUJITA-TOSHIGAKI, S. YOKOYAMA & S. NISHIMURA: Trichostatin A inhibits both *ras*-induced neurite outgrowth of PC12 cells and morphological transformation of NIH3T3 cells. *Oncogene* 10: 1119-1123, 1995
- 13) WEBB, S., C. MORRIS & J. SPRENT: Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. *Cell* 63: 1249-1256, 1990
- 14) TAKAHASHI, I., S. NAKANISHI, H. KOBAYASHI, H. NAKANO, K. SUZUKI & T. TAMAKI: Hypericin and pseudohypericin specifically inhibit protein kinase C: possible relation to their antitumor activity. *Biochem. Biophys. Res. Commun.* 165: 1201-1212, 1989
- 15) TSURUFUJI, M., B. BENACERRAF & M. SY: An antigen-specific signal is required for the activation of second-order suppressor T cells in the regulation of delayed-type hypersensitivity to 2,4,6-trinitrobenzene sulfonic acid. *J. Exp. Med.* 158: 932-945, 1983
- 16) TSUJI, N. & M. KOBAYASHI: Trichostatin C, a glucopyranosyl hydroxamate. *J. Antibiotics* 31: 939-944, 1978
- 17) COUSINS, L. S., D. GALLWITZ & B. M. ALBERTS: Different accessibilities in chromatin to histone acetylase. *J. Biol. Chem.* 254: 1716-1723, 1979
- 18) CANDIDO, E. P. M., R. REZES & J. R. DAVIE: Sodium butyrate inhibits histone deacetylation in cultured cells. *Cell* 14: 105-113, 1978
- 19) SBALY, L. & R. CHALKLEY: The effect of sodium butyrate on histone modification. *Cell* 14: 115-121, 1978
- 20) VIDALI, G., L. C. BOYA, E. M. BRADY & V. G. ALLFREY: Butyrate suppression of histone deacetylation leads to accumulation of multiacetylated forms of histones H3 and H4 and increased DNase I sensitivity of the associated DNA sequences. *Proc. Natl. Acad. Sci. U.S.A.* 75: 2239-2243, 1978
- 21) SOULEMANI, A. & C. ASSELINE: Regulation of *c-myc* expression by sodium butyrate in the colon carcinoma cell line Caco-2. *FEBS Letters* 326: 45-50, 1993
- 22) CSORDAS, A.: On the biological role of histone acetylation. *Biochem. J.* 265: 23-38, 1990
- 23) ALLFREY, V. G., R. FAULGNER & A. E. MISKEY: Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. U.S.A.* 51: 786-794, 1964